Volume 17 Number 14 1989 Nucleic Acids Research

Mechanism of incision by an apurinic/apyrimidinic endonuclease present in human placenta

Bjørn-Ivar Haukanes*, Christian-Urs Wittwer[1] and Dag Emil Helland

Laboratory of Biotechnology, University of Bergen, Bergen and [1]Department of Biochemistry, Institute of Medical Biology, University of Tromsø, 9001 Tromsø, Norway

Received April 24, 1989; Revised and Accepted June 12, 1989

ABSTRACT

An apparently homogeneous enzyme preparation, containing an apurinic/apyrimidinic (AP) endonuclease from human placenta, was by DNA sequencing analysis found to act as a class I AP-endonuclease, i.e. produce a 3'-deoxyribose and 5'-phosphomonoester nucleotide termini.

INTRODUCTION

Apurinic/apyrimidinic (AP) sites are among the most common DNA lesions (1,2). These noncoding lesions are formed by spontaneous hydrolysis, by enzymatic removal of altered bases by specific glycosylases, or by the action of chemical or physical agents (1,3,4). Such lesions are recognized by AP-endonucleases which cleave the phosphodiester bond with great specificity. The AP-endonucleaseses are present in all biological systems studied (3,7,8,9,10) and are classified according to their position of incision relative to the apurinic/apyrimidinic-site. Class I enzymes cleave at the 3'-side of the AP-site and generate a 3'-deoxyribose and a 5'-phosphomonoester nucleotide termini and probably act as β-elimination catalysts (5,6), and class II enzymes cleave at the 5'-side of the apurinic/apyrimidinic site and generate a deoxyribose-5'-phosphate at the 5'-terminus and a 3'-hydroxyl nucleotide terminus. The 3'-OH termini generated by class II enzymes can serve as potential priming sites for DNA polymerase I (*E.coli*). AP-endonucleolytic activity has previously been detected in extracts from placental tissue by Linsley et.al. (21) and purified to apparent homogenity by Shaper et.al. (22) and Wittwer et.al. (11). In this report, we present the cut-site determination of an apparently homogeneous preparation of AP-endonuclease from human placental tissue, purified by Wittwer et.al. (11). The position of incision have been studied by DNA sequencing analysis (14).

EXPERIMENTAL PROCEDURES

The AP-endonuclease preparation

Purification of the human placental AP-endonuclease was performed as described for uracil-DNA glycosylase (11). The purification include CM-Sephadex C50 chromatography, gel filtration on Sephacryl S-200, gel filtration on Biogel P-100, poly U-Sepharose 4B chromatography, and FPLC chromatography on a Mono S cation exchanger. The apparently homogeneous preparation of 'Protein 2' (M_r=26,500) isolated by Wittwer et.al. (11) was further analysed and found to act as an Mg^{++}-dependent AP-endonuclease . This AP-endonucleolytic activity was studied in the present cut-site analysis.

Preparation of DNAs

The ϕX174 RFI (replicative form I) DNA employed in the cut-site analysis was first digested

with the restriction endonuclease *Xho*I and then either labeled with (α-^{32}P)dNTPs and the Klenow fragment, or treated with calf intestinal phosphatase prior to labeling with (γ-^{32}P)ATP and T_4-polynucleotide kinase. The linearized plasmid, labeled either in both 3'-ends or in both 5'-ends, were subsequently digsted with the restriction endonucleases *Pst*I and *Hae*III. The double-digest resulted in either two 3'-labeled fragments, 273- and 166 bp in length, or two 5'-labeled fragments, 273- and 170 bp respectively. The four fragments were after this treatment only labeled in one strand. The 166 bp 3'-labeled fragment and the 170 bp 5'-labeled fragment were employed in the cut-site analysis.

DNA modification

Apurinic sites were introduced in the ^{32}P-labeled DNAs by treatment with 70% formic acid for 5 minutes at room temperature (12,13,14). Approximately one apurinic site was introduced per labeled DNA strand, and this DNA was employed as substrate for the placenta tissue apurinic/apyrimidinic endonuclease and the endonuclease III from *E.coli*.

Enzymes

Endonuclease III (*E.coli*), was kindly provided by Dr. Richard P. Cunningham, Department of Biological Sciences, State University of New York, Albany, N.Y., USA.

The placenta apurinic/apyrimidinic endonuclease was purified as described above (11). The specific activity was 44,900 units/mg. The restriction endonucleases *Pst*I and *Hae*III were from New England Biolabs and the *Xho*I restriction endonuclease and T_4-polynucleotide kinase were purchased from Amersham International. Calf intestinal phosphatase (CIP) was from P-L Biochemicals, Inc., Milwaukee.

Enzyme assays

The standard assay for the placenta apurinic/apyrimidinic endonuclease contained 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 40 mM NaCl, and 10 mM 2-mercaptoethanol. The incubation was carried out at 37°C for 30 minutes. The enzyme reaction was terminated by phenol extraction. One unit of AP-endonuclease activity is defined as the amount of enzyme required to convert 1 pmole ϕX174 RFI DNA molecules to the RFII form when incubated at 37°C for 30 minutes under standard assay conditions. The AP-endonucleolytic activity was measured by the aid of the nitrocellulose filter binding technique (15,16). The 3'-phosphatase assay mixture contained 6 units of T_4-polynucleotide kinase, 25 mM 2(N-morpholino)ethanesulfonic acid pH 6.0, 8 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 15 mM ammonium acetate. The standard assay for calf intestinal phosphatase (CIP) contained 50 mM Tris-HCl, pH 8.4, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.1 unit CIP in addition to AP-DNA, and was carried out for 2×30 minutes at 37°C.

Cut-site determination

The cut-site determination was performed as described previously (14). Sugar residues were removed by piperidine treatment (1.0 M piperidine, 20 minutes at 95°C), and 3'-phosphates were removed by the aid of the 3'-phosphatase activity of T_4-polynucleotide kinase (2−3 hrs. at 37°C). Calf intestinal phosphatase was used to remove 5'-phosphates.

RESULTS

The cut-site analysis was performed as previously described (14). The 166 bp *Xho*I-*Pst*I/*Hae*III fragment from bacteriophage ϕX174 RFI DNA, labeled with ^{32}P in one strand (3'-labeled) and containing approximately one AP-site per labeled strand, was used to analyse the 3'-side of the AP-site cleaved by the placental apurinic/apyrimidinic endonuclease (Figure 1). Two DNA sequencing lanes (G and A+G) were run alongside the analysis-lanes on the sequencing gel. The DNA containing AP-sites was first cleaved

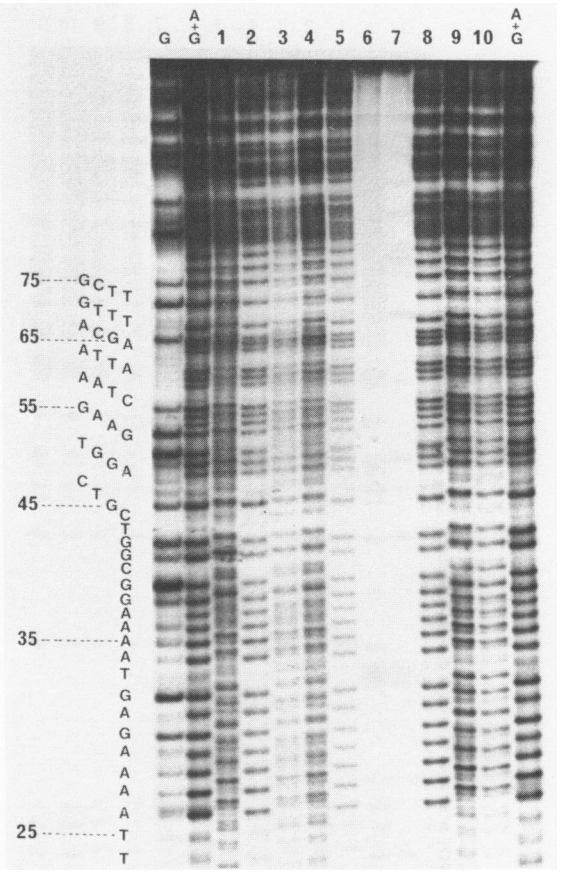

FIGURE 1. *Activity of the placental tissue apurinic/apyrimidinic endonuclease on 3'-labeled DNA*
The 166 bp *Xho*I-*Pst*I/*Hae*III fragment from ϕX174 replicative form I DNA was sequenced according to the procedure of Maxam and Gilbert (12,13). Approximately 50 ng (6×10^4cpm) DNA from the A+G sequencing reaction were used per lane, and incubated with 3 units AP-endonuclease for 30 minutes at 37°C in a 50 μl standard assay mixture. The reaction was stopped by phenol extraction and the DNA analysed on a 20% DNA-sequencing gel. The piperidine- and 5'-phosphatase modification reactions were performed as described in Experimental Procedures.
G, Gua-reaction; A+G, Gua+Ade sepcific reaction. Lanes 1−5 and 8−10 contained apurinic DNA (as A+G lane). In lane 1, piperidine- and 5'-phosphatase treatment were included; lane 5, the negative control, heat inactivated AP-endonuclease was included; lanes 2−4, incubated with the AP-endonuclease (3 units, 30 minutes at 37°C); and lanes 8−10, incubated with endonuclease III (*E.coli*) a class I AP-endonuclease. Lanes 3 and 9, enzyme digest were followed by 5'-phosphatase treatment; lanes 4 and 10, treated as in lanes 3 and 9, respectively, prior to piperidine treatment. Lanes 6 and 7 contained untreated DNA; lane 6 incubated with the AP-endonuclease and lane 7 with heat inactivated AP-endonuclease.

by the placental AP-endonuclease (Figure 1, lane 2). The DNA in this lane comigrate with the DNA with chemically cleaved AP-sites (12,13) which contain a phosphate at the 5'-end. It does not comigrate with the DNA in lane 1, where the 5'-phosphorus have been removed after chemically cleavage with piperidine. This finding indicate that the AP-

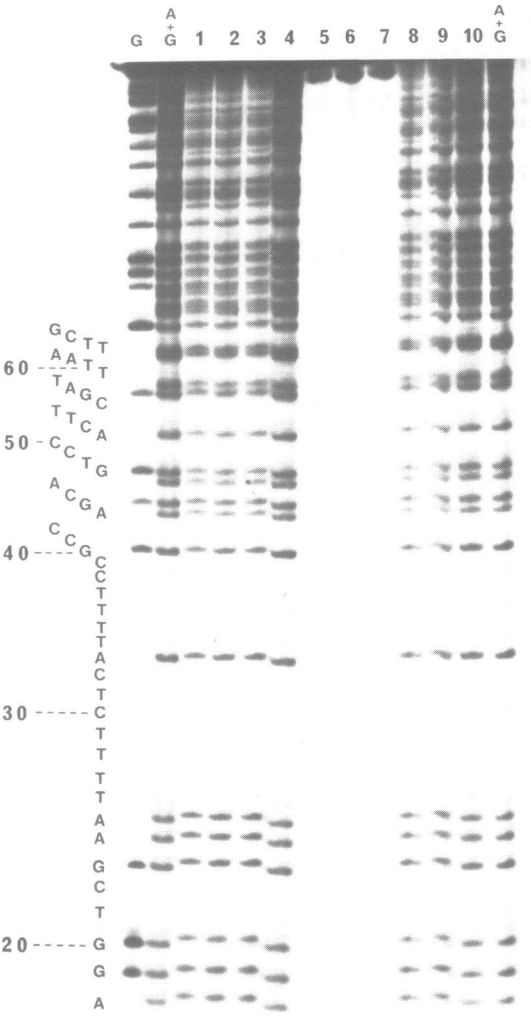

FIGURE 2. *Activity of the AP-endonuclease on 5'-labeled DNA*
The 170 bp *Xho*I-*Pst*I/*Hae*III fragment from bacteriophage ϕX174 RFI DNA was sequenced according to the procedure of Maxam and Gilbert (12,13). Approximately 70 ng (2×10^5 cpm) DNA from the A+G sequencing reaction were used per lane, and incubated with 3 units AP-endonuclease for 30 minutes at 37°C in a 50 μl standard assay mixture. The reaction was stopped by phenol extraction and the DNA analysed on a 20% denaturing polyacrylamide sequencing gel. The 3'-phosphatase- and piperidine modification reactions were performed as described in Experimental Procedures.
G, Gua-reaction; A+G, Gua+Ade specific reaction. Lanes 1−5 and 8−10 contained apurinic DNA (as A+G lane). In lane 1, piperidine- and 3'-phosphatase treatment were included; and lanes 2−4, incubated with AP-endonuclease (3 units, 30 minutes at 37°C); lane 5, the negative control, heat inactivated AP-endonuclease was included; lanes 8−10, incubated with endonuclease III (*E.coli*) (a class I AP-endonuclease). In lanes 3 and 9, enzyme digest were followed by 3'-phosphatase treatment; lanes 4 and 10, treated as in lanes 3 and 9, respectively, prior to piperidine treatment. Lanes 6 and 7 contained untreated DNA; lane 6 incubated with the AP-endonuclease and lane 7 with heat inactivated AP-endonuclease.

endonuclease in question is either a class I or a class II AP-endonuclease (14). From lane 2 to lane 3 in Figure 1, a shift in migration can be observed. The AP-DNA in lane 3 has first been cleaved with the AP-endonuclease, prior to treatment with calf intestinal phosphatase, a 5′-phosphatase. The shift in migration observed indicate that a phosphate have been removed from the 5′-end by the latter treatment. However, both class I and class II AP-endonucleases leave a phosphate at the 3′-side of the AP-site, i.e. at the 5′-end of the 3′-labeled substrate. To separate between class I and class II, the AP-DNA was treated as in lane 3 followed by piperidine treatment (lane 4). A shift between lane 3 and lane 4 would indicate a sugar residue left at the 3′-side of the AP-site. However, no shift can be observed between lane 3 and lane 4, which indicate that the placental AP-endonuclease have initially cleaved the AP-site as a class I enzyme, leaving only a phosphorus at the 3′-side of the AP-site, i.e. at the 5′-end of the 3′-labeled fragment.

Endonuclease III (*E.coli*) (17,18,19) which cleave AP-sites as a class I enzyme (20), was used in lanes 8 through 10 (Figure 1). In lane 8 the 3′-labeled AP-DNA was digested only with endonuclease III, and the DNA in this lane comigrates with the chemically cleaved AP-DNA in the A+G lanes, and the placental AP-endonuclease cleaved AP-DNA in lane 2. Lane 9 corresponds to lane 3 and lane 10 corresponds to lane 4, respectively, and both enzymes appears to act the same way i.e. cleave the AP-sites at exactly the same position.

In Figure 2, the 5′-labeled ϕX174 *Xho*I-*Pst*I/*Hae*III RFI DNA fragment was employed to analyse the 5′-side of AP-sites cleaved by the placental AP-endonuclease. As in Figure 1, DNA sequencing lanes (G, and A+G) were run alongside the analysis-lanes. Approximately one AP-site was introduced per labeled DNA strand, and cleavage of this 5′-labeled AP-DNA with the placental AP-endonuclease are displayed in lane 2 (Figure 2). The DNA in this lane does not comigrate with the chemically cleaved AP-DNA in the A+G lane. However, they do comigrate with the chemically cleaved AP-DNA which have been treated with the 3′-phosphatase activity of T_4-polynucleotide kinase (lane 1 in Figure 2). This finding indicate that the enzyme in question act either as a class I enzyme or as a class II enzyme, thus separating class I/II from III/IV (14). In lane 3, the AP-DNA was treated as in lane 2, but in addition treated with the 3′-phosphatase. No shift in migration can be observed between lane 2 and 3, which indicate that no phosphate are left at the 5′-side of the cleaved AP-sites, i.e. at the 3′-end of the 5′-labeled DNA fragment. In lane 4, the AP-DNA was treated as in lane 3 and then treated with piperidine to remove any sugar residue. A clear shift can be seen between the DNA bands in lanes 3 and 4. The DNA bands in lane 4 moves faster in the sequencing gel compared with lane 3, and comigrates with chemically cleaved AP-DNA (A+G lane). This indicate that a sugar residue has been removed from the 3′-end, i.e. the 5′-side of the AP-site, by the latter treatment. If the enzyme in question was a class II enzyme, no sugar residue would have been left at the 5′-side of the cleaved AP-sites and no shift would have been seen. The shift observed clearly indicate that the placental AP-endonuclease examined has initially cleaved the AP-DNA as a class I enzyme. This finding is identical with the results from the analysis with 3′-labeled AP-DNA (above). Furthermore, the results strongly suggest that the AP-endonuclease in question can only cleave at one side of an AP-site, the 3′-side, and that no excision takes place.

Endonuclease III (*E.coli*) has been employed as a reference enzyme also in the 5′-side analysis. Lanes 8 through 10 which contain AP-DNA cleaved with endonuclease III, corresponds to lanes 2 through 4, respectively. Figure 2 indicates that the cleavage pattern for the two enzymes are exactly the same.

DISCUSSION

Initially, the AP-endonucleolytic activity characterized in this work copurified with the uracil-DNA glycosylase described by Wittwer et.al. (11). The uracil-DNA glycosylase activity eluted in five different peaks in an isocratic region at 100 mM NaCl from a Mono S cation exchanger (FPLC) (11). A peak (named 'Protein 2' by Wittwer et.al. (11)) with no significant uracil-DNA glycosylase activity, was eluted at a somewhat higher salt concentration. When analysed by SDS-polyacrylamide gel electrophoresis and silver staining, the 'Protein 2' appeared as one major band at M_r=26,500. The data obtained also indicate that the protein is a single polypeptide chain (11). The amino acid composition and the NH_2-terminal amino acid sequence were very different from the placental uracil-DNA glycosylase (11). When analysing this 'Protein 2' fraction purified by Wittwer et.al. (11), we observed that it cleaved DNA at apurinic sites and displayed an absolute requirement for Mg^{++} (data not shown). The relative molecular weight of this AP-endonuclease correspond roughly, (or slightly smaller,) to the molecular weights of the multiple forms partially purified by Linsley et.al. (21). The requirements for divalent cations are shared with the AP-endonuclease purified to an apparently homogenous preparation from human placenta by Shaper et.al. (22). However, the latter enzyme has a slightly higher molecular weight and has been characterized to be 40% class I and 60% class II, i.e. the activity is able to cleave either 3′ (position I (24)) or 5′ (position II (24)) to the AP-site (23). This is the only activity with such a characterization among all AP-endonucleases studied so far. However, the class I activity in this preparation might be due to a contamination in the enzyme preparation. We postulate that the human placental tissue contain at least two different AP-endonucleolytic activities, one class I AP-endonuclease classified in the present paper, and one class II enzyme with a somewhat higher molecular weight (23).

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Norwegian Cancer Society (LmK). D.E.H. is a research fellow of this society.

*To whom correspondence should be addressed

REFERENCES

1. Lindahl, T. and Nyberg, B. (1972) Biochemistry, 11, 3610–3618.
2. Lindahl, T. and Karlstrøm, O. (1973) Biochemistry, 12, 5151–5154.
3. Lindahl, T. (1979) Prog. Nucl. Acids Res. Mol. Biol., 22, 135–192.
4. Dunlap, B. and Cerutti, P. (1975) FEBS Lett., 51, 188–190.
5. Bailly, V. and Verly, W.G. (1987) Biochem. J., 242, 565–572.
6. Kim, J. and Linn, S. (1988) Nucl.Acids Res., 16, 1135–1141.
7. Laval, J. (1978) Biochimie, 60, 1123–1134.
8. Lindahl, T. (1982) Ann. Rev. Biochem., 51, 61–87.
9. Hanawalt, P.C., Cooper, P.K., Ganesan, A.K., and Smith, C.A. (1979) Ann. Rev. Biochem., 48, 783–836.
10. Friedberg, E.C., Bonura, T., Radany, E.H., and Love, J.D. (1981) Enzymes that incise damaged DNA. In 'The enzymes'. (ed. P.D. Boyer), vol. 14, 251–279, Academic Press, N.Y.
11. Wittwer, C.U., Bauw, G., and Krokan, H. (1989) Biochemistry, 28, 780–784.
12. Maxam, A.M. and Gilbert, W. (1977) Proc. Nat. Acad. Sci. (USA), 74, 560–564.
13. Maxam, A.M. and Gilbert, W. (1980) Meth. Enz., 65, 499–560.
14. Haukanes, B.I., Helland, D.E., and Kleppe, K. (1988) Nucl. Acids Res., 16, 6871–6882.
15. Center, M.S. and Richardson, C.C. (1970) J. Biol. Chem., 245, 6285–6291.

16. Braun, A. and Grossman, L. (1974) Proc. Nat. Acad. Sci. (USA), 71, 1838–1842.
17. Cunningham, R.P. and Weiss, B. (1985) Proc. Nat. Acad. Sci. (USA), 82, 474–478.
18. Helland, D.E., Doetsch, P.W., and Haseltine, W.A. (1986) Mol. and Cell. Biol., 6, 1983–1990.
19. Doetsch, P.W., Helland, D.E., and Haseltine, W.A. (1986) Biochemistry, 25, 2212–2220.
20. Demple, B. and Linn, S. (1980) Nature (London), 287, 203–208.
21. Linsley, W.S., Penhoet, E.E., and Linn, S. (1977) J. Biol. Chem., 252, 1235–1242.
22. Shaper, N.L., Grafstrom, R.H., and Grossman, L. (1982) J. Biol. Chem., 257, 13455–13458.
23. Grafstrom, R.H., Shaper, N.L., and Grossman, L. (1982) J. Biol. Chem., 257, 13459–13464.
24. Mosbaugh, D.W. and Linn, S. (1980) J. Biol. Chem., 255, 11743–11752.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.